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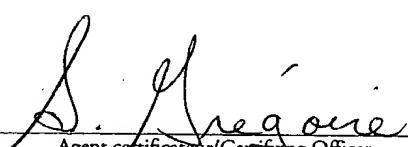
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Specification and Drawings, as originally filed, with Application for Patent Serial No:
2,219,713, on October 29, 1997 by **MCGILL UNIVERSITY**, assignee of Philippe
Séguéla and Kazimierz Babinski, for "Dna Encoding a Human Proton-Gated Ion Channel
and Uses Thereof".



Agent certificateur/Certifying Officer

April 11, 2003

Date

Canada

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O P I C  C I P O

ABSTRACT OF THE INVENTION

The present invention relates to a novel DNA sequence encoding a novel subtype of human proton-gated channel (ASIC3) ; and uses of the sequence thereof.

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DNA ENCODING A HUMAN PROTON-GATED ION CHANNEL AND USES THEREOF

BACKGROUND OF THE INVENTION

5 (a) Field of the Invention

The invention relates to a DNA sequence encoding a novel subtype of human proton-gated channel; and uses of the sequence thereof.

(b) Description of Prior Art

- 10 The neuronal excitation induced by the contact of acid on peripheral nerve endings has been linked to the activation of specific proton-sensitive cation channels expressed in primary sensory neurons of mammals (Rang et al. (1991) *Br. Med. Bull.* **47**:534-548).
- 15 The prolonged pain associated with the contact of acid on peripheral nerve endings is due to the activation of non-inactivating proton-gated channels. The duration of the acid-induced pain could neither be explained by the properties of the proton-gated channel ASIC1 cloned
- 20 from rat (Waldmann et al. (1997) *Nature* **386**:173-177) and human (Garcia-Anoveros et al. (1997) *Proc. Natl. Acad. Sci. (USA)* **94**:1459-1464) central neurons, nor by the properties of the proton-gated channel ASIC2 cloned also from rat (Waldmann et al. (1997) *Nature* **386**:173-177) and human (Price et al. (1996) *J. Biol. Chem.* **271**:7879-7882) central neurons. ASIC1 is sensitive to pH 6.5 and lower but inactivates Waldmann et al. (1997) *Nature* **386**:173-177). ASIC2 is sensitive to pH lower than 6 and inactivates rapidly.
- 25 It would be highly desirable to be provided with the primary structure of non-inactivating proton-activated channels from human sensory neurons and means for their functional expression.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide the primary structure and functional expression of a subtype of non-inactivating proton-gated channel from 5 human sensory neurons.

Another aim of the present invention is to provide a DNA sequence encoding a novel subtype of human proton-gated channel.

In accordance with the present invention there 10 is provided an isolated nucleic acid molecule which consists essentially of the nucleotide sequence depicted in Figs. 1A and 1B.

The isolated nucleic acid molecule of the present invention encode a peptide consisting essentially 15 of the amino acid sequence depicted in Figs. 1A and 1B.

In accordance with the present invention there is provided a vector, preferably an expression vector, selected from the group consisting of plasmids, phage, retrovirus, baculovirus and integration elements, which 20 include the isolated nucleic acid molecule of the present invention.

In accordance with the present invention there is provided an isolated nucleic acid molecule, which is capable of hybridizing to the isolated nucleic acid 25 molecule depicted in Figs. 1A and 1B, wherein the hybridization occurs at about 35°C to about 65°C and in 5X SSPC and 50% formamide or equivalent hybridization conditions thereto.

In accordance with the present invention there 30 is provided a method of using the isolated nucleic acid molecule depicted in Figs. 1A and 1B, or a sequence which hybridizes under stringent condition to the sequence depicted in Figs. 1A and 1B, to produce a peptide consisting essentially of the amino acid sequence

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depicted in Figs. 1A and 1B, which comprises the steps of:

- a) transforming a host with a DNA sequence capable of encoding the peptide;
- 5 b) incubating the host under conditions which allows the sequence to be express;
- c) isolating the peptide from the host; and
- d) recording or imaging the activity of the peptide from the host.

10 The preferred host is selected from the group consisting of bacteria, yeast, fungi, mammalian cells, plant cells, and insect cells.

In accordance with the present invention there is provided a method of using the peptide encoded by 15 the amino acid sequence depicted in Figs. 1A and 1B or domains of the peptide, to produce antibodies, which comprises the steps of:

- a) immunizing a host with the peptide or domains of the peptide for a time sufficient for an 20 immunogenic reaction to occur; and
- b) isolating antibodies from the immunized host.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B illustrate the primary structure of the cDNA (1732 bases) encoding the full-length 25 human ASIC3 (hASIC3) channel subunit. The coding region of 531 amino acids encoded in the mRNA corresponds to nucleotides 22 to 1614;

Fig. 2 illustrates the recording of non-inactivating cationic current induced by strong acid (pH 4.0) 30 in Xenopus oocytes injected with hASIC3 clone alone in pCDNA3 vector; and

Fig. 3 illustrates the recording of non-inactivating cationic current induced by weak acid (pH 6.5)

in *Xenopus* oocytes co-injected with hASIC3 clone and rat P2X2 clone both in pCDNA3 vector.

DETAILED DESCRIPTION OF THE INVENTION

5

Molecular cloning of hASIC3 and in vitro translation

Using the TBLASTN algorithm (Altschul et al. (1990) *J. Mol. Biol.* 215:403-410), virtual screening of the dbEST database with the conserved domain LXTFP AVTLCNXN of ASIC1 and ASIC2 subunits led to the identification of two human fetal brain EST sequences coding for a novel proton-gated channel subunit (EST IDs # AA449579 and AA429417). The clone tagged by EST #AA449579 was sequenced on both strands and was shown to encode a full-length human proton-gated channel subunit (Figs. 1A and 1B). Characteristic natural and unique restriction sites for *Cla*I, *Sma*I, *Sac*I, *Nco*I, *Xho*I and *Xba*I are indicated by arrowheads.

This hASIC3 clone was transferred into the HindIII-NotI sites of eukaryotic vector pcDNA3 (Invitrogen) for CMV-driven heterologous expression in HEK-293 cells and *Xenopus* oocytes. Supercoiled hASIC3 plasmid was used for in vitro translation using the TnT system (Promega) with T7 RNA polymerase and [³⁵S]-Cysteine according to manufacturer's specifications. The apparent molecular weight of monomeric hASIC3 subunits was 57±3 kiloDaltons, in excellent agreement with the molecular weight of 58.8 kiloDaltons calculated from the predicted primary sequence of the clone.

Functional expression of hASIC3 in *Xenopus* oocytes

Oocytes surgically removed from mature *Xenopus laevis* frogs were treated 2 hrs at room temperature with type II collagenase (Gibco-BRL) in Barth's solution under agitation. Selected stage IV-V oocytes were defolliculated manually before nuclear microinjection

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(Séguéla et al. (1996) *J. Neurosci.* 16:448-455) of 10 ng cDNA of hASIC3 in pcDNA3 vector. After 2-4 days of expression at 19°C in Barth's solution containing 10µg/ml gentamycin, oocytes were recorded in two-
5 electrode voltage-clamp configuration using a OC-725B amplifier (Warner Inst.). Signals were acquired and digitized at 500 Hz using a Macintosh IIci equipped with an A/D card NB-MIO16XL (National Instruments) then traces were post-filtered at 100 Hz in Axograph (Axon
10 Instruments). Acidic solutions titrated at room temperature in Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂ in 10 mM HEPES were applied during 10 seconds on oocytes by perfusion in constant flow (10 ml/min). During recording, oocyte
15 membrane was clamped at V_h=-100 mV.

There is shown in Fig. 2 the recording of non-inactivating cationic current induced by strong acid (pH 4.0) in Xenopus oocytes injected with hASIC3 clone alone in pcDNA3 vector. These data demonstrate that
20 hASIC3 alone can associate in functional homomeric cation channels.

There is shown in Fig. 3 the recording of non-inactivating cationic current induced by weak acid (pH 6.5) in Xenopus oocytes co-injected with hASIC3 clone and rat P2X2 clone both in pCDNA3 vector. These data demonstrate that the co-expression of hASIC3 and rat P2X2 changes the pH sensitivity of homomeric hASIC3 or leads to the formation of heteromeric pH-sensitive
25 channels.

30 The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

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EXAMPLE I

Functional expression of recombinant ASIC3 channels in eukaryotic cells

5 Development of analgesic therapeutical compounds used for the clinically-relevant pharmacological modulation, inhibition or activation of human ASIC3 channels and homologous receptors.

10

EXAMPLE II

Uses of antibodies directed against human ASIC3 channel subunits

15 Polyclonal or monoclonal antibodies can be directed against a bacterial fusion protein containing predicted antigenic domains of hASIC3 subunit, or can be directed against peptides from the predicted amino acid sequence of hASIC3 subunit.

Potential uses:

20 Regional and cellular *in situ* immunolocalization of mammalian ASIC3 channels in cells naturally or artificially expressing ASIC3 channels.

25 Immunoprecipitation of mammalian ASIC3 channels for purification of ASIC3 channels and associated proteins, quantitation of ASIC3 channels and associated proteins.

Western blot detection of mammalian ASIC3 channels from cells naturally or artificially expressing ASIC3 channels.

30 Identification of members of the mammalian ASIC gene family using antibodies for screening expression cDNA libraries.

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EXAMPLE III

Uses of human ASIC3 DNA sequence

Identification of novel members of the
5 mammalian ASIC channel family as potential therapeutic
targets using hASIC3 channel subunit sequence for the
design of nucleic acid hybridization probe or PCR
degenerate oligonucleotide primers. While the
invention has been described in connection with
10 specific embodiments thereof, it will be understood
that it is capable of further modifications and this
application is intended to cover any variations, uses,
or adaptations of the invention following, in general,
the principles of the invention and including such
15 departures from the present disclosure as come within
known or customary practice within the art to which the
invention pertains and as may be applied to the
essential features hereinbefore set forth, and as
follows in the scope of the appended claims.

WE CLAIM:

1. An isolated nucleic acid molecule encoding peptides consisting essentially of the amino acid sequences depicted in Figs. 1A and 1B.
2. The isolated nucleic acid of claim 1, wherein said sequence consists essentially of the nucleotide sequence depicted in Figs. 1A and 1B.
3. The isolated nucleic acid of claim 1 or 2, wherein said sequence further comprises a vector selected from the group consisting of plasmids, phages, virus and integration elements.
4. The isolated nucleic acid of claim 3, wherein said vector is an expression vector.
5. An isolated nucleic acid molecule, which is capable of hybridizing to the isolated nucleic acid molecule of claim 1 or 2, wherein said hybridization occurs at about 35°C to about 65°C and in 5X SSPC and 50% formamide or equivalent hybridization conditions thereto.
6. A method of using the isolated nucleic acid molecule depicted in Figs. 1A and 1B, or a sequence which hybridizes under stringent condition to said sequence depicted in Figs. 1A and 1B, to produce peptides consisting essentially of the amino acid sequences depicted in Figs. 1A and 1B, which comprises the steps of:
 - a) transforming a host with a DNA sequence capable of encoding said peptide;

- b) incubating said host under conditions which allows said sequence to be express;
- c) isolating said peptide from said host; and
- d) recording or imaging the activity of said peptide from said host.

7. The method of claim 6, wherein said host is selected from the group consisting of bacteria, yeast, fungi, mammalian cells, plant cells, and insect cells.

8. A method of using the peptide encoded by the amino acid sequence depicted in Figs. 1A and 1B or domains of said peptide, to produce antibodies, which comprises the steps of:

- a) immunizing a host with said peptide or domains of said peptide for a time sufficient for an immunogenic reaction to occur; and
- b) isolating antibodies from said immunized host.

human ASIC3

TCGCACGACG CGGTTCTGGC CATGAAGCCC ACCTCAGGCC CAGAGGAGC CCGGCGGCAG	60
M K P T S G P E E A R R Q	13
MetLysPro ThrSerGlyP roGluGluAl aArgArgGln	
CCCTCGGACA TCCCGGTGTT CGCCAGAAC TGCTCGATGC ACGGGCTGGG CCACGTCTTC	120
P S D I R V F A S N C S M H G L G H V F	33
ProSerAspI leArgValPh eAlaSerAsn CysSerMetH isGlyLeuG1 yHisValPhe	
GGGCCAGGCA GCCTGAGCCT GCGCCGGGG ATGTGGGCAG CGGCCGTGGT CCTGTCAGTG	180
G P G S L S L R R G M W A A A V V L S V	53
GlyProGlyS erLeuSerLe uArgArgGly MetTrpAlaA laAlaValVa lLeuSerVal	
GCCACCTTCC TCTACCAGGT GGCTGAGAGG GTGCGCTACT ACAGGGAGTT CCACCAACAG	240
A T F L Y Q V A E R V R Y Y R E F H H Q	73
AlaThrPheL euTyrGlnVa lAlaGluArg ValArgTyrT yrArgGluPh eHisHisGln	
ACTGCCCTGG ATGAGCGAGA AAGCCACCGG CTCGTCTTCC CGGCTGTCAC CCTGTGCAAC	300
T A L D E R E S H R L V F P A V T L C N	93
ThrAlaLeuA spGluArgG1 uSerHisArg LeuValPheP roAlaValTh rLeuCysAsn	
ATCAACCCAC TGCGCCGCTC GCGCTTAACG CCCAACGACC TGCACGGCC TGGGTCTGCG	360
I N P L R R S R L T P N D L H W A G S A	113
IleAsnProL euArgArgSe rArgLeuThr ProAsnAspL euHisTrpAl aGlySerAla	
CTGCTGGGCC TGGATCCCCG AGAGCACGCC GCCTTCTGC GCGCCCTGGG CCGGCCCCCT	420
L L G L D P A E H A A F L R A L G R P P	133
LeuLeuGlyL euAspProAl aGluHisAla AlaPheLeuA rgAlaLeuG1 yArgProPro	
GCACCGCCCG GCTTCATGCC CAGTCCCACC TTTGACATGG CGCAACTCTA TGCCCGTGCT	480
A P P G F M P S P T F D M A Q L Y A R A	153
AlaProProG lyPheMetPr oSerProThr PheAspMetA laGlnLeuTy rAlaArgAla	
GGGCACTCCC TGGATGACAT GCTGCTGGAC TGTCGCTTCC GTGGCCAACC TTGTGGGCC	540
G H S L D D M L L D C R F R G Q P C G P	173
GlyHisSerL euAspAspMe tLeuLeuAsp CysArgPheA rgGlyGlnPr oCysGlyPro	
GAGAACTTCA CCACGATCTT CACCCGGATG GGAAAGTGCT ACACATTTAA CTCTGGCGCT	600
E N F T T I F T R M G K C Y T P N S G A	193
GluAsnPheT hrThrIlePh eThrArgMet GlyLysCysT yrThrPheAs nSerGlyAla	
GATGGGGCAG AGCTGCTCAC CACTACTAGG GGTGGCATGG GCAATGGCT GGACATCATG	660
D G A E L L T T T R G G M G N G L D I M	213
AspGlyAlaG luLeuLeuTh rThrThrArg GlyGlyMetG lyAsnGlyLe uAspIleMet	
CTGGACGTGC AGCAGGAGGA ATATCTACCT GTGTGGAGGG ACAATGAGGA GACCCCGTT	720
L D V Q Q E E Y L P V W R D N E E T P F	233
LeuAspValG InGlnGluG1 uTyrLeuPro ValTrpArgA spAsnGluG1 uThrProPhe	
ClaI	
GAGGTGGGGA TCCGAGTGCA GATCCACAGC CAGGAGGAGC CGCCCATCAT CGATCAGCTG	780
E V G I R V Q I H S Q E E P P I I D Q L	253
GluValGlyI leArgValG1 nIleHisSer GlnGluGluP roProIleI1 eAspGlnLeu	
SmaI	
GGCTTGGGG TGTCCTGGG CTACCAAGACC TTTGTTCTT GCCAGCAGCA GCAGCTGAGC	840
G L G V S P G Y Q T F V S C Q Q Q Q L S	273
GlyLeuGlyV alSerProG1 yTyrGlnThr PheValSerC ysGlnGlnG1 nGlnLeuSer	

Fig. 1A

human ASIC3

TTCCTGCCAC	CGCCCTGGGG	CGATTGCAGT	TCAGCATCTC	TGAACCCCAA	CTATGAGCCA	900
F L P P	P W G	D C S	S A S L	N P N	Y E P	293
PheLeuProP	roProTrpG1	yAspCysSer	SerAlaSerL	euAsnProAs	nTyrGluPro	
GAGCCCTCTG	ATCCCCTAGG	CTCCCCCAGC	CCCAGCCCCA	GCCCTCCCTA	TACCCTTATG	960
E P S D	P L G	S P S	P S P S	P P Y	T L M	313
GluProSerA	spProLeuG1	ySerProSer	ProSerProS	erProProTy	rThrLeuMet	
GGGTGTCGCC	TGGCCTGCCA	AACCCGCTAC	GTGGCTCGGA	AGTGCGGCTG	CCGAATGGTG	1020
G C R L	A C E	T R Y	V A R K	C G C	R M V	333
GlyCysArgL	euAlaCysG1	uThrArgTyr	ValAlaArgL	ysCysGlyCy	sArgMetVal	
TACATGCCAG	GCGACGTGCC	AGTGTGCAGC	CCCCAGCAGT	ACAAGAACTG	TGCCCACCCG	1080
Y M P G	D V P	V C S	P Q Q Y	K N C	A H P	353
TyrMetProG	lyAspValPr	oValCysSer	ProGlnGlnT	yrLysAsnCy	sAlaHisPro	
GCCATAGATG	CCATCCTTCG	CAAGGACTCG	TGCGCCTGCC	CCAACCCGTG	CCCCAGCACG	1140
A I D A	I L R	K D S	C A C P	N P C	A S T	373
AlaIleAspA	laIleLeuAr	gLysAspSer	CysAlaCysP	roAsnProCy	sAlaSerThr	

NcoI

SacI

CGCTACGCCA	AGGAGCTCTC	CATGGTGCAG	ATCCCGAGCC	GCGCCGCCGC	GCGCTTCCTG	1200
R Y A K	E L S	M V R	I P S R	A A A	R F L	393
ArgTyrAlaL	ysGluLeuSe	rMetValArg	IleProSerA	rgAlaAlaAl	aArgPheLeu	
GCCCCGAAAGC	TCAACCGCAG	CGAGGCCCTAC	ATCCGGGAGA	ACGTGCTGGC	CCTGGACATC	1260
A R K L	N R S	E A Y	I A E N	V L A	L D I	413
AlaArgLysL	euAsnArgSe	rGluAlaTyr	IleAlaGluA	snValLeuAl	aLeuAspIle	
TTCTTGAGG	CCCTCAACTA	TGAGACCGTG	GAGCAGAAGA	AGGCCTATGA	GATGTCAGAG	1320
F F E A	L N Y	E T V	E Q K K	A Y E	M S E	433
PhePheGluA	laLeuAsnTy	rGluThrVal	GluGlnLysL	ysAlaTyrG1	uMetSerGlu	
CTGCTTGGTG	ACATTGGGGG	CCAGATGGGC	CTTTTCATCG	GGGCCAGCCT	GCTCACCATC	1380
L L G D	I G G	Q M G	L F I G	A S L	L T I	453
LeuLeuGlyA	spIleGlyG1	yGlnMetGly	LeuPheIleG	lyAlaSerLe	uLeuThrIle	

XbaI

CTCGAGATCC	TAGACTACCT	CTGTGAGGTG	TTCCGAGACA	AGGTCTGGG	ATATTTCTGG	1440
L E I L	D Y L	C E V	F R D K	V L G	Y F W	473
LeuGluIleL	euAspTyrLe	uCysGluVal	PheArgAspL	ysValLeuG1	yTyrPheTrp	
AACCGACAGC	ACTCCCAAAG	GCACTCCAGC	ACCAATCTGC	TTCAGGAAGG	GCTGGGCAGC	1500
N R Q H	S Q R	H S S	T N L L	Q E G	L G S	493
AsnArgGlnH	isSerGlnAr	gHisSerSer	ThrAsnLeuL	euGlnGluG1	yLeuGlySer	
CATCGAACCC	AAGTTCCCCA	CCTCAGCCTG	GGCCCCAGAC	CTCCCACCCC	TCCCTGTGCC	1560
H R T Q	V P H	L S L	G P R P	P T P	P C A	513
HisArgThrG	InValProHi	sLeuSerLeu	GlyProArgP	roProThrPr	oProCysAla	

XbaI

GTCACCAAGA	CTCTCTCCGC	CTCCCACCGC	ACCTGCTACC	TTGTCACACA	GCTCTAGACC	1620
V T K T	L S A	S H R	T C Y L	V T Q L	.	531
ValThrLysT	hrLeuSerAl	aSerHisArg	ThrCysTyrl	euValThrG1	nLeu...	
TGCTGCTCTGT	GTCCTCGGAG	CCCCGCCCTG	ACATCCTGGA	CATGCCCTAGC	CTGCACGTAG	1680
CTTTTCGGTC	TTCACCCCAA	ATAAAGTCCT	AATGCATCAA	AAAAAAA	AA	1732

Fig. 1B

**Non-desensitizing pH-sensitive inward current in Xenopus Oocytes
microinjected with hASIC3**

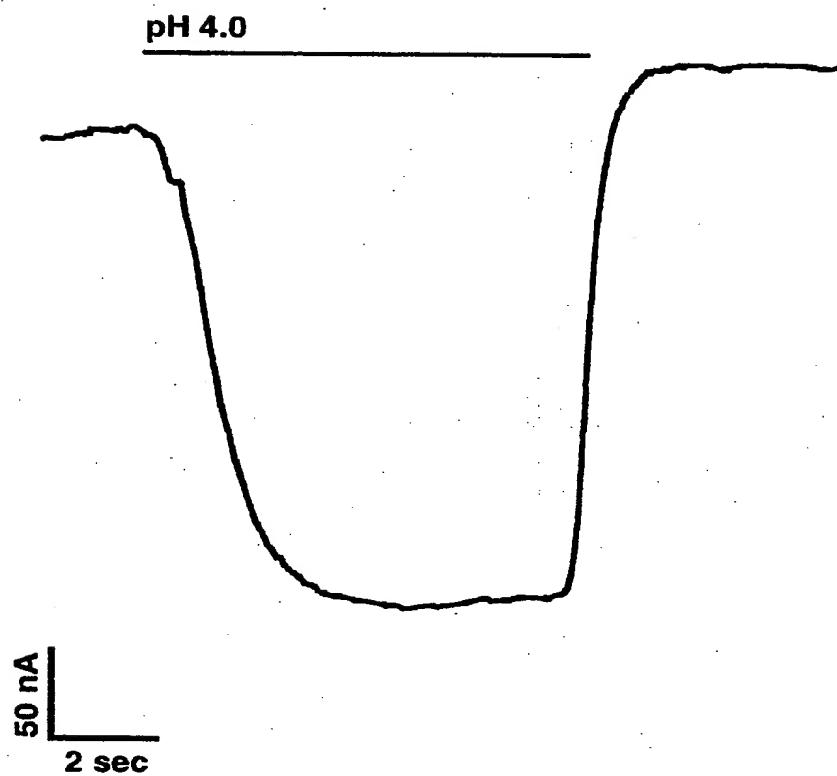


Fig. 2

Non-desensitizing pH-sensitive current in Xenopus oocytes
microinjected with human ASIC3 + rat P2X2

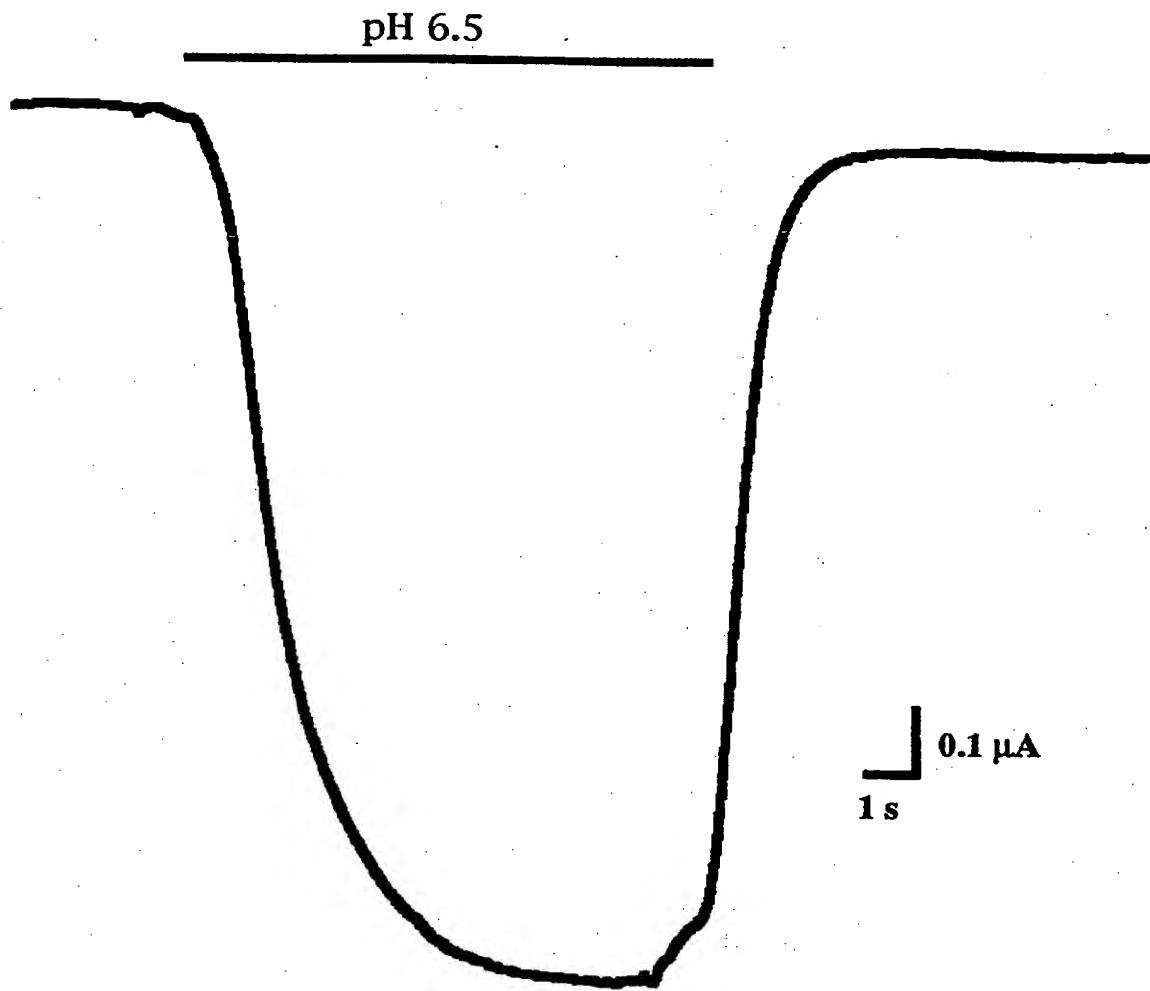


Fig. 3

